Actinoplanes rhizophilus sp. nov., an actinomycete isolated from the rhizosphere of Sansevieria trifasciata Prain

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A novel actinomycete, designated strain NEAU-A-2T, was isolated from the rhizosphere soil of Sansevieria trifasciata Prain collected from Heilongjiang province, north-east China. The taxonomic status of this organism was established using a polyphasic approach. The isolate formed irregular sporangia containing motile spores on the substrate mycelium. The whole-cell sugars were xylose and galactose. The predominant menaquinones were MK-9(H10), MK-9(H2), MK-10(H2) and MK-10(H4). The major fatty acids were iso-C15:0, iso-C16:0 and anteiso-C15:0. The polar lipids were diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylinositol, three unidentified phospholipids and an unidentified glycolipid. 16S rRNA gene sequence similarity studies showed that strain NEAU-A-2T belongs to the genus Actinoplanes with the highest sequence similarities to Actinoplanes globisporus NBRC 13912T (97.7% 16S rRNA gene sequence similarity), Actinoplanes ferrugineus IMSNU 22125T (97.5%), Actinoplanes toevensis MN07-A0368T (97.2%) and Actinoplanes rishiriensis NBRC 108556T (97.2%); similarities to type strains of other species of this genus were <97%. Two tree-making algorithms showed that strain NEAU-A-2T formed a distinct clade with A. globisporus NBRC 13912T and A. rishiriensis NBRC 108556T. However, low DNA–DNA relatedness values allowed the isolate to be differentiated from the above-mentioned two species of the genus Actinoplanes. Moreover, strain NEAU-A-2T could also be distinguished from the most closely related species by morphological and physiological characteristics. Therefore, in conclusion, isolate NEAU-A-2T represents a novel species of the genus Actinoplanes, for which the name Actinoplanes rhizophilus sp. nov. is proposed. The type strain is NEAU-A-2T (=CGMCC 4.7133T=DSM 46672T).

The genus Actinoplanes was first described by Couch (1950) for actinomycetes that produced motile spores with spherical, cylindrical, digitate, lobate, bottle, flask-shaped or very irregular sporangia by tufts of polar flagella at the ends of sporangioaphores on substrate mycelium. The detailed phenotypic and chemotaxonomic analysis of the genus was first provided by Goodfellow et al. (1990), and a comprehensive phylogenetic analysis of the genus was given by Tamura & Hatano (2001). At the time of writing, the genus Actinoplanes comprises 37 species with validly published names (http://www.bacterio.net/actinoplanes.html), including the recently described Actinoplanes nipponensis (Wink et al., 2014), Actinoplanes lutulentus (Gao et al., 2014a) and Actinoplanes siamensis (Suriyachakun et al., 2013). During an investigation exploring potential sources of novel species and novel natural products, strain NEAU-A-2T was isolated from rhizosphere soil of Sansevieria trifasciata Prain. In this study, we performed polyphasic taxonomy on this strain, and propose that strain NEAU-A-2T represents a novel species of the genus Actinoplanes. Strain NEAU-A-2T was isolated from the rhizosphere soil of Sansevieria trifasciata Prain collected from Heilongjiang province, north-east China (45° 30’ N 127° 06 ’ E). The strain was isolated using the standard dilution plate
method and grown on humic acid-vitamin agar (HV; Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l\(^{-1}\)) and nalidixic acid (20 mg l\(^{-1}\)). After 21 days of aerobic incubation at 28 °C, a colony was transferred and purified on oatmeal agar [International Streptomyces Project (ISP) 3 medium] (Shirling & Gottlieb, 1966) and maintained as glycerol suspensions (20 %, v/v) at -80 °C. The type strains of *Actinoplanes globisporus* and *Actinoplanes rishiriensis* were purchased from the NITE Biological Resource Center (NBRC) and cultured under the same conditions for comparative analysis.

Morphology of the sporangia was observed by scanning electron microscopy (S-3400N; Hitachi) using cultures grown on Bennett’s agar (per litre distilled water: 1 g yeast extract, 1 g beef extract, 2 g NZ amine type A, 10 g glucose and 20 g agar; pH 7.3) for 20 days. Spore motility was assessed by light microscopic (ECLIPSE E200; Nikon) observation of cells suspended in 1 mM phosphate buffer (pH 7.0). Cultural characteristics were determined by growth on Bennett’s agar and ISP media 2–7 (Shirling & Gottlieb, 1966) at 28 °C for 14 days. The ISCC-NBS colour charts were used to determine the names and designations of colony colours (Kelly, 1964). Growth at different temperatures (4, 10, 15, 18, 22, 28, 30, 32, 35 and 40 °C) was determined on Bennett’s agar after incubation for 14 days. Growth tests for pH range [pH 4–10 in 1 unit intervals, using the buffer system described by Xie et al. (2012)] and NaCl tolerance [0–7 % (w/v) in 1 % intervals] were determined in Bennett’s broth at 28 °C for 14 days on a rotary shaker. Production of catalase, esterase and urease were tested as described by Smibert & Krieg (1994). The utilization of sole carbon and nitrogen sources, decomposition of cellulose, hydrolysis of starch and aesculin, reduction of nitrate, peptonization of milk, liquefaction of gelatin and production of H2S were examined as described previously (Gordon et al., 1974; Yokota et al., 1993).

Freeze-dried cells used for chemotaxonomic analysis were obtained from cultures grown in Bennett’s broth on a rotary shaker for 7 days at 28 °C. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of dianimopimelic acid in the peptidoglycan were derivatized according to McKerrow et al. (2000), and analysed by HPLC using an Agilent TC-C18 column (250 × 4.6 mm i.d. 5 µm) with a mobile phase consisting of 0.05 mol l\(^{-1}\) phosphate buffer pH 7.2 (0.2 M NaH₂PO₄/0.2 M Na₂HPO₄, 28 : 72, v/v) and acetoni trile in a ratio of 85 : 15 (v/v), at a flow rate of 0.5 ml min\(^{-1}\). The peak detection used an Agilent G1321A fluorescence detector with a 365 nm excitation and 455 nm longpass emission filters. The N-acyl group of muramic acid in peptidoglycan was determined by the method of Uchida et al. (1999). The whole-cell sugars were analysed according to the procedures developed by Lechevalier & Lechevalier (1980). Polar lipids in cells were extracted and identified using the method of Minnikin et al. (1984). Menaquinones were extracted from freeze-dried biomass and purified according to Collins (1985). Extracts were analysed by a HPLC-UV method using an Agilent Extend-C18 column (150 × 4.6 mm, i.d. 5 µm), typically at 270 nm. The mobile phase was acetoni trile/propyl alcohol (60 : 40, v/v), the flow rate was set to 1.0 ml min\(^{-1}\) and the run time was 60 min. The chromatographic column was controlled at 40 °C (Wu et al., 1989). Mycolic acids were checked by the acid methanolysis method as described previously (Minnikin et al., 1980). Fatty acid methyl esters were extracted from the biomass as described by Gao et al. (2014b) after culture in Bennett’s broth at 28 °C for 14 days on a rotary shaker. The fatty acid methyl esters were analysed by GC-MS using the method of Xiang et al. (2011).

Extraction of chromosomal DNA and PCR-mediated amplification of the 16S rRNA gene were carried out using a standard procedure (Kim et al., 2000). The PCR product was purified and cloned into the vector pMD19-T (Takara) and sequenced by using an Applied Biosystems DNA sequencer (model 3730XL). An almost full-length 16S rRNA gene sequence (1512 nt) of strain NEAU-A-2\(^{T}\) was obtained and aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using CLUSTAL X 1.83 software. The EzTaxon-e server (Kim et al., 2012) was employed to identify the phylogenetic neighbours and calculate the pairwise 16S rRNA gene sequence similarities. Phylogenetic trees were generated with the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms using MEGA software version 5.05 (Tamura et al., 2011) with 1369 nt. The stability of the clades in the trees was appraised using a bootstrap value with 1000 repeats (Felsenstein, 1985). A distance matrix was generated using Kimura’s two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

The G + C content of the genomic DNA was determined by the thermal denaturation (Tm) method as described by Mandel & Marmur (1968), and *Escherichia coli* JM109 was used as the reference strain. DNA–DNA relatedness tests between strain NEAU-A-2\(^{T}\) and *Actinoplanes globisporus* NBRC 13912\(^{T}\) and *Actinoplanes rishiriensis* NBRC 108556\(^{T}\) were carried out as described by De Ley et al. (1970), under consideration of the modifications described by Huss et al. (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multichannel changer and a temperature controller with in situ temperature probe (Varian). The concentration and purity of DNA samples were determined by measuring the optical density at 260, 280 and 230 nm. The DNA samples used for hybridization were diluted to OD\(_{260}\) around 1.0 using 0.1 × SSC (saline sodium citrate buffer), then were sheared using a JY92-II ultrasonic cell disruptor (ultrasonic time 3 s and interval time 4 s for 90 cycles). The DNA renaturation rates were determined in 2 × SSC at 70 °C.
Morphological observation of a 20-day-old culture of strain NEAU-A-2\textsuperscript{T} grown on Bennett’s agar revealed that it had characteristics typical of the genus *Actinoplanes*. Strain NEAU-A-2\textsuperscript{T} produced branched, non-fragmenting substrate hyphae, which bore irregular sporangia (4.5–5.8 μm) and sporangium releasing motile spores (Fig. S1, available in the online Supplementary Material). The sporangiospores were motile. Strain NEAU-A-2\textsuperscript{T} showed good growth on ISP 2 and Bennett’s agar, moderate growth on ISP 3, ISP 6 and ISP 7 agar, and poor growth on ISP 4 and ISP 5 agar (Table S1). The colour of colonies on different media was yellow to orange. Diffusible pigments or melanin were not formed on the tested media. Growth of strain NEAU-A-2\textsuperscript{T} occurred at pH 6.0–8.0 and in the presence of 0–1 % (w/v) NaCl, with optimum growth at pH 7.0. The temperature range for growth was 15–35 °C, with optimum growth at 28 °C. Detailed physiological and biochemical properties are presented in the species description.

Strain NEAU-A-2\textsuperscript{T} contained meso-diaminopimelic acid and glycine in the cell-wall hydrosylates. Whole-cell hydrosylates contained galactose and xylose. The acyl type of cell-wall peptidoglycan was glycolyl. The polar lipid profile consisted of diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylinositol, three unidentified phospholipids and an

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**Fig. 1.** Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (1369 nt, by omitting unaligned regions) showing the relationship between strain NEAU-A-2\textsuperscript{T} and species of the genus *Actinoplanes*. Asterisks indicate branches that were also recovered using the maximum-likelihood algorithm. Bootstrap values >50 % (based on 1000 replications) are shown at branch points. *Dactylosporangium aurantiacum* DSM 43157\textsuperscript{T} was used as the outgroup. Bar, 0.005 substitutions per nucleotide position.
unidentified glycolipid (Fig. S2). The predominant menaquinones were MK-9(H10) (31.1 %), MK-9(H2) (29.8 %), MK-10(H2) (27.4 %) and MK-10(H4) (11.7 %). The fatty acids were iso-C15 : 0 (25.1 %), iso-C16 : 0 (17.8 %), anteiso-C15 : 0 (12.3 %), C17 : 1ω7c (6.6 %), C16 : 0 (6.5 %), anteiso-C17 : 0 (6.0 %), C17 : 0 (4.9 %), iso-C14 : 0 (4.8 %), C15 : 0 (4.7 %), C18 : 0 (3.4 %), C18 : 1ω7c (2.8 %), C18 : 1ω9c (2.5 %), C16 : 1ω7c (1.3 %), C17 : 0 cyclo (1.0 %) and C14 : 0 (0.3 %) (Table S2). Mycolic acids were not detected. The DNA G+C content was 73.3 ± 0.3 mol%. All the morphological characteristics and chemotaxonomic data showed that strain NEAU-A-2T should be assigned to the genus Actinoplanes.

Based on EzTaxon-e analysis, the 16S rRNA gene sequence (1512 nt) showed that strain NEAU-A-2T belongs to the genus Actinoplanes, with the highest 16S rRNA gene sequence similarities to A. globisporus NBRC 13912T (97.7 %) 16S rRNA gene sequence similarity), Actinoplanes ferrugineus IMSNU 22125T (97.5 %), Actinoplanes toensis MN07-A0368T (97.2 %) and A. rishiriensis NBRC 108556T (97.2 %). The 16S rRNA gene sequence similarities to type strains of other species of this genus were <97 %. However, phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain NEAU-A-2T formed a distinct phylectic line with A. globisporus NBRC 13912T and A. rishiriensis NBRC 108556T, an association that was supported by a bootstrap value of 91 % in the neighbour-joining tree (Fig. 1) and also recovered with the maximum-likelihood algorithm. DNA–DNA hybridization was employed to further clarify the relatedness between strain NEAU-A-2T and A. globisporus NBRC 13912T and A. rishiriensis NBRC 108556T; the DNA–DNA relatedness values between strain NEAU-A-2T and A. globisporus NBRC 13912T and A. rishiriensis NBRC 108556T were 55.1 ± 1.2 % and 53.2 ± 1.8 %, respectively. These values were below the threshold value of 70 % recommended by Wayne et al. (1987) for assigning strains to the same genomic species. In addition, strain NEAU-A-2T could be clearly distinguished from A. globisporus NBRC 13912T and A. rishiriensis NBRC 108556T based on phenotypic characteristics, as summarized in Table 1 and Table S1. Strain NEAU-A-2T had different colonial characteristics on various media compared with the most closely related species. The novel strain grew at 15 °C, while the most closely related species could not, and the isolate could not tolerate 2 % NaCl, whereas A. globisporus NBRC 13912T and A. rishiriensis NBRC 108556T could. For chemotaxonomic characteristics, members of the genus Actinoplanes contained tetrahydrogenated menaquinones with nine isoprene units [MK-9(H10)] as the predominant isoprenologue and usually smaller proportions of MK-9(H4) and MK-9(H2) (Goodfellow et al., 1990). However, strain NEAU-A-2T contains MK-9(H10) as the predominant menaquinone and less MK-9(H2), MK-10(H2) and MK-10(H4). The differences in fatty acids between strain NEAU-A-2T and the two reference strains are shown in Table S2. Other phenotypic characteristics that differentiated strain NEAU-A-2T from A. globisporus NBRC 13912T and A. rishiriensis NBRC 108556T included liquefaction of gelatin, hydrolysis of starch, reduction of nitrate, decomposition of urea and patterns of carbon and nitrogen source utilization.

In conclusion, it is evident from the genotypic, chemotaxonomic and phenotypic data that strain NEAU-A-2T represents a novel species of the genus Actinoplanes, for which the name Actinoplanes rhizophilus sp. nov. is proposed.

**Description of Actinoplanes rhizophilus sp. nov.**

Actinoplanes rhizophilus (rhi.zo’phi.lus. Gr. n. rhiza a root; Gr. adj. philos loving; N.L. masc. n. rhizophilus root-loving).

Aerobic, Gram-stain-positive actinomycete that forms well-developed substrate mycelia, which carry irregular sporangia when grown on Bennett’s agar. Motile sporangiospores are formed. Grows well on ISP 2 and Bennett’s agar, moderately on ISP 3, ISP 6 and ISP 7 agar, and poorly on ISP 4 and ISP 5 agar. No diffusible pigments or melanin are detected on any of the tested media. Positive for reduction of nitrate and hydrolysis of aesculin, but negative for decomposition of cellulose and urea, production of esterase, catalase and H2S, hydrolysis of starch, liquefaction of gelatin and peptonization of milk. L-Arabinose, D-galactose, D-glucose, inositol, lactose, maltose, D-mannitol, D-mannose, raffinose, L-rhamnose, D-ribose, D-sorbitol and sucrose are utilized as sole carbon sources, but D-fructose and D-xyllose are not.

### Table 1. Differential phenotypic properties of strain NEAU-A-2T and the phylogenetically most closely related strains

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<th>Characteristic</th>
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<td>Hydrolysis of starch</td>
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<td>Decomposition of urea</td>
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<td>Reduction of nitrate</td>
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<td>Liquefaction of gelatin</td>
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<td>+</td>
<td>−</td>
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<tr>
<td>Tolerance of NaCl (%; w/v)</td>
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<td>3</td>
<td>2</td>
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<tr>
<td>Growth pH</td>
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<td>6–10</td>
<td>5–8</td>
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<tr>
<td>Growth temperature (°C)</td>
<td>15–35</td>
<td>20–28</td>
<td>20–35</td>
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Utilization of:

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<th>Carbohydrates</th>
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<td>−</td>
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<tr>
<td>L-Serine</td>
<td>−</td>
<td>+</td>
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Strains: 1, NEAU-A-2T; 2, A. globisporus NBRC 13912T; 3, A. rishiriensis NBRC 108556T. +, Positive; −, negative. Data were obtained in this study.
Glycine is utilized as a sole nitrogen source, but L-alanine, L-arginine, L-asparagine, L-aspartic acid, creatine, L-glutamic acid, L-glutamine, L-serine, L-threonine and L-tyrosine are not. Tolerates up to 1% NaCl and grows at temperatures between 15 and 35°C (optimum 28°C) and at pH 6.0–8.0 (optimum pH 7.0). Cell-wall hydrolysates contain meso-diaminopimelic acid and glycine. The whole-cell sugars are galactose and xylose. The acyl type of cell-wall peptidoglycan is glycolyl. Mycolic acids are absent. The major menaquinones are MK-9(H₂), MK-9(H₄), MK-10(H₂) and MK-10(H₄). The polar lipid profile consists of diphosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylinositol, three unidentified phospholipids and an unidentified glycolipid. The major cellular fatty acids are iso-C₁₅ : ₀, iso-C₁₆ : ₀ and anteiso-C₁₅ : ₀.

The type strain is NEAU-A-2²³ (=CGMCC 4.7133²³=DSM 46672²³), which was isolated from the rhizosphere soil of Sansevieria trifasciata Prain collected from Heilongjiang province, north-east China. The DNA G+C content of the type strain is 73.3 ± 0.3 mol%.

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References


